

Cerebral serotonin release correlates with [^{11}C]AZ10419369 PET measures of 5-HT_{1B} receptor binding in the pig brain

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Abstract

Positron emission tomography (PET) can, when used with appropriate radioligands, non-invasively capture temporal and spatial information about acute changes in brain neurotransmitter systems. We here evaluate the 5-HT_{1B} receptor partial agonist PET radioligand, [^{11}C]AZ10419369, for its sensitivity to detect changes in endogenous cerebral serotonin levels, as induced by different pharmacological challenges. To enable a direct translation of PET imaging data to changes in brain serotonin levels, we compared the [^{11}C]AZ10419369 PET signal in the pig brain to simultaneous measurements of extracellular serotonin levels with microdialysis after various acute interventions (saline, escitalopram, fenfluramine). The interventions increased the cerebral extracellular serotonin levels to two to six times baseline, with fenfluramine being the most potent pharmacological enhancer of serotonin release. The interventions induced a varying degree of decline in [^{11}C]AZ10419369 binding in the brain, consistent with the occupancy competition model. The observed correlation between changes in the extracellular serotonin level in the pig brain and the 5-HT_{1B} receptor occupancy indicates that [^{11}C]AZ10419369 binding is sensitive to changes in endogenous serotonin levels to a degree equivalent to that reported of [^{11}C]raclopride to dopamine, a much used approach to detect in vivo change in cerebral dopamine.

Keywords

Positron emission tomography, serotonin, brain imaging, kinetic modelling, neurosurgery

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Introduction

Several studies have investigated PET radioligands for their ability to be displaced by endogenously released serotonin. Such PET radioligands would be an important tool to investigate disorders associated to specific serotonin subtypes and serotonergic dysfunction as well as pharmacological treatments targeting these receptors. Unfortunately, the outcomes in humans have not convincingly shown the expected effect of pharmacological interventions supposed to increase brain interstitial serotonin.^{1,2}

The 5-HT_{1B} receptor is one of 14 mammalian serotonin receptor subtypes; each receptor is associated with specific physiological impact and has a distinct regional distribution in the brain.^{3,4} The 5-HT_{1B} receptor is located on the terminals both presynaptically, where it acts as a 5-HT autoreceptor regulating serotonin release, and postsynaptically as a heteroreceptor, where it interacts with other neurotransmitter systems

such as acetylcholine, glutamate, GABA and dopamine.³ The receptor is involved in regulation of several physiological functions such as increased secretion of corticosterone and prolactin, sexual function, appetite, thermoregulation and locomotion.³ Further, the 5-HT_{1B} receptor has been associated to many

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neuropsychiatric disorders such as depression, anxiety and OCD.⁵ From a clinical perspective, modulation of serotonin at the 5-HT_{1B} receptor has a high therapeutic potential. Hence, the ability to image changes in serotonin would constitute an important contribution to unveil disease mechanisms and to identify therapeutic options.

PET studies with the 5-HT_{1B} receptor radioligands [¹¹C]AZ10419369^{6,7} or [¹¹C]P943⁸ in humans and non-human primates have investigated the extent of radioligand displacement from the receptor by an increase in endogenous serotonin elicited by a serotonergic challenge. In non-human primates, studies^{6,7} demonstrate a dose-dependent decrease in radioligand binding across brain regions following intervention with fenfluramine (a strong serotonin releaser) or a high dose of the serotonin reuptake inhibitor (SSRI) escitalopram. In humans, however, an increased radioligand binding was observed following a clinical relevant dose of escitalopram.⁷ Similarly, in another study in humans, the cerebral binding of the 5-HT_{1A} receptor PET radioligand [¹¹C]CUMI-101 was also found to increase following a citalopram⁹ challenge although no change was found in another [¹¹C]CUMI-101 study.¹⁰ This unexpected finding in humans was interpreted in both studies as being caused by autoreceptor function, with 5-HT_{1B} or 5-HT_{1A} autoreceptor stimulation inhibiting serotonin release.

Such paradoxical SSRI-induced effects on interstitial brain serotonin level have not been observed in microdialysis studies, which is the gold standard procedure to investigate changes in neurotransmitter levels. Microdialysis studies demonstrate that 5-HT_{1B} autoreceptor function can dampen or even cancel out the anticipated SSRI-induced increase in cerebral serotonin level in a region-dependent way, but it does not decrease the serotonin level compared to baseline.^{11–13} However, microdialysis studies to our knowledge have so far only investigated SSRI doses manifold above clinically relevant doses. Therefore, the conflicting outcomes of the human and the non-human primate PET studies may be explained by species differences or differences in the doses applied since a high SSRI dose would be expected to lead to a larger increase in brain interstitial serotonin levels.

The aim of the present study was to measure the effect of pharmacological challenges – also in clinically relevant doses – on the serotonin level by cerebral microdialysis and to correlate this to the change in the non-displaceable binding potential (BP_{ND}) of the 5-HT_{1B} receptor partial agonist radioligand [¹¹C]AZ10419369 in the pig brain.

We hypothesized that pharmacologically induced acute increases in extracellular brain serotonin level were associated with a decline in [¹¹C]AZ10419369 5-HT_{1B} receptor binding, in consistency with the competition model.¹

Material and methods

Animals

We used 10 9–10-week old Danish Landrace female pigs weighing 22 ± 1.3 kg (mean ± SD) in this study. All animal experiments were performed in accordance with the European Communities Council Resolves on 22 September 2010 (2010/63/EU) and approved by the Danish Veterinary and Food Administration's Council for Animal Experimentation (Journal No. 2012-15-2934-00156), and is in compliance with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines). We performed tranquilization, anesthesia, intubation, installation of arterial and venous intravenous lines in the morning, monitoring and sacrifice of animals late afternoon was done as previously described.¹⁴ On the day of investigation, we performed surgery with implantation of microdialysis guide cannulas and CMA 12 metal-free microdialysis probe (CMA Microdialysis) bilaterally into the medial prefrontal cortex (mPFC) using a well-validated procedure to allow full embedment of the active part of the microdialysis probe in only the grey matter of cortex. The procedural details are given in our previous study¹⁵ and a schematic diagram of the events on the investigation day in Figure 1.

Study design

Both PET scans were conducted on the same day as within scan challenge with saline (scan 1) and escitalopram or fenfluramine (scan 2). Upon insertion of

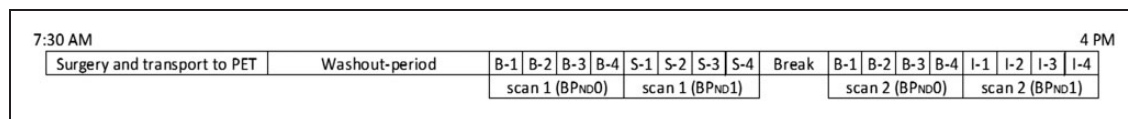


Figure 1. Microdialysis measurements were obtained with a time resolution of 15 min at baseline (B1–B4) prior to intervention with saline (S1–S4) or escitalopram/fenfluramine (I1–I4).

microdialysis probes bilaterally in the mPFC, we placed the pig in the PET scanner and allowed a 2 h-washout period to obtain steady serotonin baseline level. Two PET scans, each lasting 120 min, were conducted 30 min apart. We administered the pharmacological interventions intravenously 56.5 min after injection of the radioligand with the aim to acutely increase the extracellular serotonin levels. The interventions consisted of either saline (controls, $n=10$), escitalopram 0.28 mg/kg (selective serotonin reuptake inhibitor (SSRI), $n=5$) or fenfluramine 0.5 mg/kg (serotonin releaser, $n=5$). The investigators were not blinded to the interventions but the pigs were randomized.

In pilot experiments prior to a previous study,¹⁵ we tested the effect of three different doses of fenfluramine; 0.05 mg/kg, 0.5 mg/kg and 2.0 mg/kg, but when 2 mg/kg was given, the pig started to shiver and showed a large increase in vital signs, so the injection was interrupted after $\frac{3}{4}$ had been given. Since the dose of 0.05 mg/kg did not induce any significant alterations in microdialysate 5-HT levels, we chose to give a dose of 0.5 mg/kg. This dose has shown to elicit a 9–11-fold increase in serotonin level consistent with the spectrum that allows comparison of changes in BP_{ND} at an 8-fold increase in neurotransmitter level such as reported for [11C]raclopride to dopamine.¹⁶

Microdialysis and serotonin measurements

The probes had a membrane length of 4 mm and a molecular weight cut-off value of 20 kDa. They were perfused with a standard Ringer solution (147 mM NaCl, 4 mM KCl and 2.3 mM CaCl₂, adjusted to pH 6.5) at a flow rate of 1.0 μ L/min. From both probes, we collected 15-min time-series of samples of extracellular fluid to be analyzed off-line for monoamines by HPLC. After a 2 h-washout period, we collected 20 samples with 15 min interval. The microdialysate samples were quickly frozen on dry ice and stored in a -80°C freezer until further analyzed.

We determined the relative changes in serotonin concentrations in the dialysate samples by HPLC with electrochemical detection. The column was a Prodigy 3 μ ODS (3) C18 (DA 2 mm \times 100 mm, particle size 3 μ m, Phenomenex, Torrance, California, USA). The mobile phase consisted of 55 mM sodium acetate, 1 mM octanesulfonic acid, 0.1 mM Na₂EDTA and 8% acetonitrile, adjusted to pH 3.2 with 0.1 M acetic acid, and was degassed with an on-line degasser. Ten microliters of the dialysate sample were injected, and the flow rate was 0.15 mL/min. The electrochemical detection was done with an amperometric detector (Antec Decade, Antec, Leiden, the Netherlands) with a glassy carbon electrode set at 0.8 V, with an Ag/AgCl reference electrode.

The output of the HPLC was recorded by the program "LC solution" (Shimadzu, Columbia, Maryland, USA), which also was used to calculate the peak areas. We determined the baseline level on the basis of the mean peak area obtained by the HPLC from the four samples preceding the pharmacological intervention of each PET scan. We used the baseline level to calculate the relative change in the serotonin levels of the four samples following the pharmacological intervention during the remaining period of each PET scan. In 19 of the performed 20 pig scans, stable baseline serotonin levels were achieved on one or both sides. In one pig, stable baseline level was not reached at the saline intervention and therefore this microdialysis data were excluded from the saline intervention analyses.

$$\text{Occupancy } (O) = \frac{\Delta F_{NT}}{K_{NT} + F_{NT} + \Delta F_{NT}} \quad (1)$$

According to the competition model,¹ there are three factors, which determine the ability of a tracer to detect changes in synaptic neurotransmission: affinity of the neurotransmitter for its receptor, K_{NT} , the basal neurotransmitter concentration in the interstitial fluid, F_{NT} , and the challenge-induced change in neurotransmitter concentration, ΔF_{NT} .

PET scanning protocol

PET scans were obtained in list mode with a high resolution research tomograph (HRRT) scanner, with the pig in the prone position. [¹¹C]AZ10419369 was given as an intravenous bolus injection and data acquisition began at the time of injection. The synthesis and radiochemical labeling of [¹¹C]AZ10419369 has previously been described.¹⁷

The average injected radioactivity of [¹¹C]AZ10419369 was 466 MBq (range, 399–510 MBq; $n=20$) and the average injected mass was 1.75 μ g (range 0.17–9.58 μ g; $n=20$) with no significant difference between scans (paired t -test). To ensure that the pharmacological challenges did not induce changes in blood [¹¹C]AZ10419369 time activity curves (TAC), we measured arterial whole blood radioactivity, in the beginning continuously for 20 min using an ABSS autosampler (Allogg Technology, Mariefred, Sweden) counting coincidences in a lead-shielded detector, and later blood samples were manually drawn at 2.5, 5, 10, 20, 30, 50, 70 and 90 min after injection and the radioactivity in whole blood and plasma samples was measured in a well counter (Cobra 5003, Packard Instruments, PerkinElmer, Skovlunde, Denmark) that was cross-calibrated to the HRRT scanner and autosampler. Radiolabeled parent

compound and metabolites were measured in plasma using HPLC with online radioactivity detection as previously described.¹⁸

Quantification of PET data

The [¹¹C]AZ10419369 HRRT PET data were acquired in 3D list mode and reconstructed using a 3D-OSEM-PSF algorithm¹⁹ with MAP-TR attenuation correction²⁰ into a dynamic image dataset consisting of 44 frames of increasing length (6 × 10, 6 × 20, 4 × 30, 9 × 60, 6 × 120, 4 × 180, 2 × 240, 1 × 300, 1 × 360, 1 × 420, 1 × 540, 1 × 780, 1 × 1380, 1 × 660 s). Images consisted of 207 planes of 256 × 256 voxels of 1.22 × 1.22 × 1.22 mm³. For each pig, a time-weighted average image was calculated including all frames. This image was used for automatic atlas labeling of the individual brain PET images.

Our in-house pig atlas²¹ was made from transferring atlas labels from a recently published MRI-based pig atlas with 178 segmented regions²² to an average PET template made of PET and MR images in pigs obtained from a recently published study with the PET radioligand 5-HT_{2A} receptor agonist [¹¹C]Cimbi-36.¹⁵

Further processing of the [¹¹C]AZ10419369 PET scans in this study was done by generating an average PET scan, which was cropped and co-registered to our in-house pig atlas using the Flirt algorithm from the FSL software package (FSL 5.0.8, FMRIB Software Library, Release 5.0 (c) 2012, The University of Oxford, UK). The estimated 12 parameter affine transformation matrix was then used for transforming the atlas labels into individual pig spaces.

Each coregistration was verified by visual inspection before extraction of TACs from the following volumes of interest (VOIs) combined from the atlas labels: neocortex (28.0 cc) (frontal cortex (4.9 cc), somatosensory-motor cortex (5.9 cc), occipital cortex (5.6 cc), insula (2.8 cc) and temporal cortex (3.8 cc)), thalamus (1.4 cc), striatum (2.0 cc) and hippocampus (0.8 cc). We used cerebellum (4.4 cc) as the reference region. TACs for these VOIs were extracted from the dynamic PET dataset.

We estimated the 5-HT_{1B} receptor binding of [¹¹C]AZ10419369 using the extended simplified reference tissue model (ESRTM)²³ before and after the serotonergic challenge given at 56.5 min. This would allow a 30-s administration interval until splitting of the dataset at 57 min, which we in pilot studies found gave stable BP_{ND0/1} estimates before and after challenge. Frames acquired at time interval 0–57 min formed the basis for calculation of BP_{ND0} and frames acquired at time interval 57–120 min for BP_{ND1}.

The relative decrease in BP_{ND} in the VOI was calculated as

$$\% \Delta BP_{ND} = -O = 100 * \frac{BP_{ND1} - BP_{ND0}}{BP_{ND0}} \quad (2)$$

To reject outliers, the standard coefficient of variance (COV) was calculated for all regional BP_{NDs} quantified by the ESRTM model. Data with COV larger than 15% were excluded, and regions with excluded data ($n < 5$) were not included in further analysis.

Statistical analysis

Similar to our previous study in pigs with [¹¹C]Cimbi-36,¹⁵ we correlated the occupancy as measured with PET with the highest peak increase (of the left or right microdialysis probe) in extracellular 5-HT levels. Unless performed with very time-consuming methods²⁴ incompatible with the use of short-lived radioisotopes and the need to conduct two PET studies, microdialysis experiments do not generate absolute serotonin concentrations. Instead, we assumed a fairly stable baseline concentration of the pig cerebral interstitial fluid serotonin of about 1.7 nM, equal to what has been found in mice.^{25–29} ΔF_{NT} was then computed as 1.7 nM times the relative peak increase in serotonin level minus 1.7 nM. The data were fitted to the competition model given in equation (1) with a non-linear regression analysis as previously described.¹⁵ We used the Wald Runs-Test for randomness to test if the curve fitted by non-linear regression to the occupancy model deviated from the data.

The mean increase in interstitial serotonin level (5-HT), as measured by microdialysis and the mean percentage (%) change in BP_{ND} of the PET scans, calculated by equation (2), was estimated in the three conditions: saline ($n = 10$), escitalopram ($n = 5$) and fenfluramine ($n = 5$). The change in BP_{ND} (PET) and 5-HT (microdialysis) was analyzed post hoc for significance between saline and either two interventions (Wilcoxon signed rank test) for differences in each region of interest.

Our study design with a within-scan saline challenge in the first scan, followed by a second scan where the intervention was given 56.5 min after the radioligand injection allowed for an assessment of the reproducibility of [¹¹C]AZ10419369 binding, both within the individual scan and between the first part of the two scans. We performed test-retest analysis of BP_{ND0} (PET 1 and 2) and BP_{ND0/1} (PET 1) in neocortex. First, we tested for significant order effect (Wilcoxon signed-rank test). Then, we determined the intraclass correlation coefficients (ICC) modelled as a two-way mixed ANOVA with absolute agreement and average measurement in a within-subject design. ICC above 0.80 is considered good and ICC above 0.90 is considered excellent.

Lastly, we calculated the required sample size for each intervention of escitalopram and fenfluramine to reach significance level of 0.05 with a power of 0.8 given the effect size determined by group parameters of mean \pm SD (BP_{ND0} and BP_{ND1}) and ICC for the neo-cortex region.

Results

In vivo microdialysis

Fenfluramine produced a powerful increase in extracellular serotonin level to a peak $580 \pm 286\%$ ($n=5$) relative to baseline level (100%) following the within-scan pharmacological challenge. Escitalopram produced a peak increase in serotonin to $233 \pm 120\%$ ($n=5$) and saline intervention did not change the serotonin level in the pig brain ($n=9$) relative to baseline level (Figure 2).

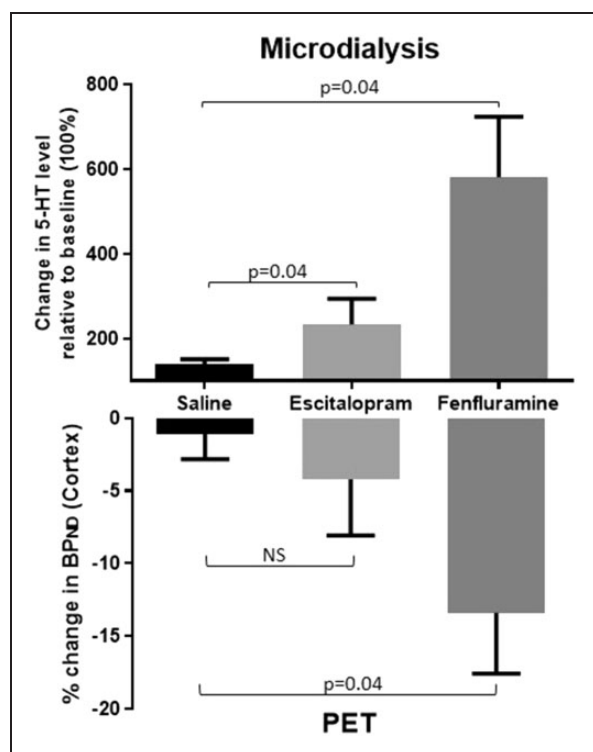


Figure 2. Changes in brain interstitial 5-HT levels (upper panel) and BP_{ND} (lower panel) following a within-scan challenge of saline ($n=10$), escitalopram ($n=5$), or fenfluramine ($n=5$). The upper panel shows the changes in the peak interstitial cerebral 5-HT level relative to baseline (100%) \pm SEM as measured by microdialysis in the mPFC. The lower panel shows the relative decrease in BP_{ND} as measured by PET in the pig brain neocortex. Changes were statistically evaluated by comparing the escitalopram and fenfluramine interventions to saline with Wilcoxon signed-rank test.

The time course of the challenge-induced increase in extracellular serotonin level is given in Figure 3.

In vivo PET imaging

With PET, we measured a significant decrease in BP_{ND} of [^{11}C]AZ10419369, corresponding to an increase in interstitial serotonin level induced by escitalopram and fenfluramine (Figure 2). Saline interventions did not induce a change in BP_{ND} measures. The BP_{ND0} , BP_{ND1} and the relative change (%) in BP_{ND} of [^{11}C]AZ10419369 in the neocortex after saline, escitalopram or fenfluramine interventions are given for all pigs individually in the supplementary material (Table 1). The average binding potential of all VOIs is listed in Table 1 in the three conditions. A significant change in binding between groups was observed in the occipital cortex ($p=0.01$) and neocortex ($p=0.02$) (Kruskal–Wallis test).

5-HT versus 5-HT_{1B} receptor occupancy

The fenfluramine-induced changes in 5-HT level were significantly increased compared to saline, as measured by both PET and microdialysis, while the escitalopram-induced changes in 5-HT level was only significantly higher when measured by microdialysis (Figure 2).

The corresponding measures of the PET occupancy and the F_{NT} were fitted to equation (1) and the observed correlation did not deviate significantly from the occupancy model (Figure 4). The affinity of the neurotransmitter for its receptor, K_{NT} , was computed to 74 ± 27 nM, about 43 times higher than the baseline brain interstitial serotonin concentration. Figure 4 shows that when brain interstitial serotonin

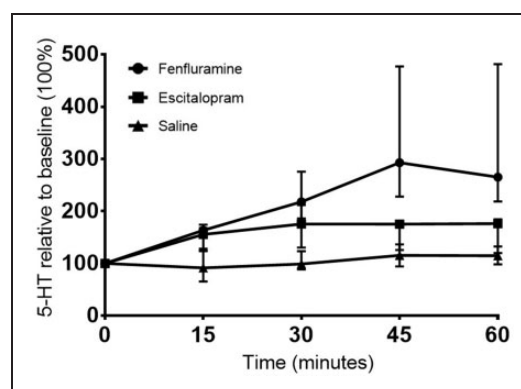


Figure 3. The median time course of the increase in extracellular cerebral 5-HT level after a pharmacological challenge of saline, escitalopram or fenfluramine administered at time 0 min. The increase in 5-HT level is presented relative to baseline (100%) with median \pm quartiles.

Table 1. Non-displaceable BP_{ND} s from the two PET scans, and % change in BP_{ND0} and BP_{ND1} , as calculated by equation (2).

Volumes of interest	BP_{ND0} (mean \pm SD)	BP_{ND1} (mean \pm SD)	ΔBP_{ND} (%) (mean \pm SD)	BP_{ND} 0/1	BP_{ND} 0/0
Saline ($n = 10$)				ICC	
Cortex (total)	0.66 ± 0.10	0.66 ± 0.13	-1 ± 5	0.98	0.94
Frontal	0.57 ± 0.11	0.54 ± 0.13	-5 ± 7	0.97	0.92
Somatosensory	0.60 ± 0.10	0.58 ± 0.14	-4 ± 8	0.96	0.95
Occipital	0.78 ± 0.11	0.74 ± 0.12	-5 ± 3	0.97	0.84
Insula	0.73 ± 0.10	0.76 ± 0.14	5 ± 8	0.91	0.87
Temporal	0.71 ± 0.12	0.72 ± 0.14	1 ± 6	0.97	0.95
Thalamus	0.90 ± 0.14	0.95 ± 0.22	4 ± 10	0.90	0.92
Dorsal striatum	1.49 ± 0.20	1.59 ± 0.20	7 ± 5	0.85	0.80
Escitalopram ($n = 5$)				Required sample size	
Cortex (total)	0.71 ± 0.10	0.68 ± 0.12	-4 ± 8	11	19
Frontal	0.64 ± 0.12	0.60 ± 0.13	-6 ± 12	8	16
Somatosensory	0.65 ± 0.11	0.60 ± 0.13	-8 ± 9	8	9
Occipital	0.79 ± 0.11	0.73 ± 0.10	-7 ± 6	5	11
Insula	0.81 ± 0.09	0.83 ± 0.15	2 ± 9	126	149
Temporal	0.76 ± 0.10	0.72 ± 0.12	-5 ± 7	8	11
Thalamus	0.98 ± 0.11	1.03 ± 0.23	4 ± 13	67	63
Dorsal striatum	1.64 ± 0.23	1.76 ± 0.30	8 ± 12	17	21
Fenfluramine ($n = 5$)					
Cortex (total)	0.64 ± 0.09	0.56 ± 0.12	-13 ± 8	5	6
Frontal	0.56 ± 0.11	0.47 ± 0.14	-18 ± 10	5	6
Somatosensory	0.58 ± 0.11	0.50 ± 0.14	-15 ± 10	6	6
Occipital	0.73 ± 0.07	0.61 ± 0.11	-16 ± 8	4	5
Insula	0.70 ± 0.11	0.64 ± 0.12	-8 ± 9	8	11
Temporal	0.66 ± 0.11	0.57 ± 0.15	-13 ± 8	5	6
Thalamus	0.95 ± 0.20	0.89 ± 0.22	-7 ± 8	24	20
Dorsal striatum	1.51 ± 0.12	1.48 ± 0.19	-2 ± 6	110	131

Note: The intercorrelation coefficient (ICC) is given for both within-PET scan (BP_{ND} 0/1) and between-PET scans (BP_{ND} 0/0) along with the required sample size given a power of 0.8, significance level of 0.05 and the effect size determined by the mean $BP_{ND} \pm SD$ and the ICC.

level increases 8-fold, [^{11}C]AZ10419369 decreases by 15% (CI7, 24%).

When we compute the data to the model with AUC instead of peak, we find that K_{NT} is 27 nM (instead of 74 nM), and the data still conform to the model. For example, a 8-fold increase in serotonin level would be associated with a 32% (rather than 15%) decline in BP_{ND} .

Test-retest analyses of [^{11}C]AZ10419369 PET measures

In neocortex, the [^{11}C]AZ10419369 had a very small test-retest variability of $-1 \pm 5\%$ (within scan) in $BP_{ND0/1}$ (PET scan 1) and $2 \pm 9\%$ (between scans) in

BP_{ND0} (PET scan 1/2) and the corresponding ICC values were 0.98 and 0.94. We did not observe a consistent order effect (Wilcoxon signed-rank test, matched pairs). The required sample size to statistically identify a fenfluramine associated 13% decline in BP_{ND} in neocortex is $n = 5$ and to find an escitalopram associated 4% decline in BP_{ND} is $n = 11$ (Table 1).

Discussion

We here show first, that intravenous injections of escitalopram and fenfluramine both induce an acute increase in the cerebral interstitial fluid concentration of serotonin in the brain. Secondly, we show that

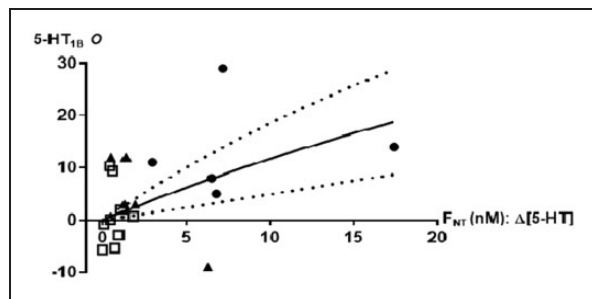


Figure 4. Relationship between relative increase in brain interstitial 5-HT levels (ΔF_{NT}) and neocortical 5-HT_{1B} receptor occupancy O (%) with 95% confidence intervals. Interventions include saline (square), escitalopram (filled triangle), and fenfluramine (filled circle). Data are based on 10 pigs with two PET scans and are fitted with nonlinear regression analysis according to equation (1), resulting in a K_{NT} of 74 nM.

the interventions induce a varying degree of decline in [¹¹C]AZ10419369 binding in the brain, consistent with the occupancy competition model. The observed correlation between changes in the extracellular serotonin level in the pig brain and the 5-HT_{1B} receptor occupancy indicates that [¹¹C]AZ10419369 binding is sensitive to changes in endogenous serotonin levels.

The pharmacological interventions induced an average 2-fold (escitalopram 0.28 mg/kg) and 6-fold (fenfluramine 0.5 mg/kg) increase in cerebral serotonin release compared to baseline level in the mPFC in the pig brain. In order to compare the outcome of our study with previously reported clinical studies,^{7,9,10} we deliberately chose to use a clinically relevant dose of escitalopram. However, somewhat unexpectedly, compared to the high dose of escitalopram (2 mg/kg) given in our previous pig study, it does not seem that using a clinically relevant dose of 0.28 mg/kg is associated with substantially less serotonin release.¹⁵ The fenfluramine-induced changes in cerebral interstitial serotonin were in line with our previous combined microdialysis and PET study in pigs with the 5-HT_{2A} receptor agonist [¹¹C]-Cimbi36¹⁵ as well as microdialysis studies in rats³⁰ and non-human primates.³¹

Depending on the pharmacological intervention, the change in 5-HT_{1B} receptor binding as measured by the PET radioligand [¹¹C]AZ10419369 ranged between an average 4% (escitalopram) and 16% (fenfluramine). A statistically significant decrease in BP_{ND} was only found in brain areas with high binding (the occipital cortex) or when a large brain volume was encountered (entire neocortex), suggesting that good count statistics is important to reveal smaller changes in BP_{ND}. The paired data on microdialysis and [¹¹C]AZ10419369 (Figure 4) comply with the competition model supporting that [¹¹C]AZ10419369 PET is sensitive to changes in

endogenous serotonin levels when the serotonin release is sufficiently high.

As speculated by Finnema et al.,³² regional differences in SERT, 5-HT_{1B} auto- and heteroreceptor densities may impact the BP_{ND} by separate mechanisms. High-density SERT regions may express a higher SSRI-induced increase in synaptic serotonin level as compared to low-density SERT regions, which may produce a less or no decrease in BP_{ND}. Given that the regional relationship between 5-HT_{1B} auto- and heteroreceptor densities is largely unknown, it is difficult to predict in which regions one can expect to see the highest sensitivity to changes in synaptic serotonin.

K_{NT} varies across brain regions and species, but in order to relate to equation (1), we assumed a fixed value for interstitial fluid serotonin. K_{NT} would change accordingly, but an 8-fold increase in serotonin level would still result in a 15% decrease in BP_{ND}. Based on the data shown in Figure 4, we estimated K_{NT} (the affinity of serotonin to the 5-HT_{1B} receptor) to be 74 nM. For comparison, K_{NT} of [¹¹C]-Cimbi36 is 14.3 nM¹⁵ and the concentration of ³H-5-HT that labels 50% of cloned human 5-HT_{1B} receptors is 0.6–15.8 nM (PDSP K_i database). We find it relevant to compare the sensitivity of [¹¹C]AZ10419369 to serotonin with that of [¹¹C]-raclopride to dopamine, since the latter is an often used approach to detect in-vivo change in cerebral dopamine, but also to the sensitivity of [¹¹C]-Cimbi36 and [¹⁸F]altanserin to detect changes in serotonin. From microdialysis studies in non-human primates, it is known that when amphetamine 0.3 mg/kg is given intravenously, the interstitial dopamine concentration increases approximately 8-fold.^{33–36} In non-human primates, an 8-fold increase in dopamine results in a decrease in [¹¹C]-raclopride BP_{ND} of 13%.¹⁶ This is consistent with findings in humans, where intravenous amphetamine 0.2–0.3 mg/kg results in a decrease in striatal BP_{ND} of 15.5%¹⁶ or 13%.^{37–39} With the 5-HT_{2A} agonist receptor [¹¹C]-Cimbi36 in pigs,¹⁵ we have recently demonstrated that a pharmacological challenge of fenfluramine and escitalopram induces a serotonergic response compatible with the competition mode 1,¹ and an 8-fold increase in the cerebral interstitial serotonin level corresponds to a 46% change in occupancy. Here we demonstrate (Figure 4) that an 8-fold increase in interstitial serotonin results in a decrease in [¹¹C]AZ10419369 BP_{ND} of 15% (CI 7, 25%), equivalent to the sensitivity of [¹¹C]-raclopride to dopamine, but three times less sensitive than [¹¹C]-Cimbi36 is to changes in the serotonin level. We here show that [¹¹C]AZ10419369 is twice as sensitive to competition from serotonin as [¹¹F]altanserin was in a human study,⁴⁰ following a single dose of dexfenfluramine (40 mg). Moreover, whereas [¹¹C]-raclopride can only be used to reliably assess dopamine changes in

striatum, both [^{11}C]AZ1041936 and [^{11}C]Cimbi36 can – due to the widespread and relatively uniform distribution of the 5-HT_{1B}R and 5-HT_{2A}R – theoretically be used to determine regional serotonin changes in the entire brain. It is an important feature of a PET radioligand to be able to identify changes also in smaller brain areas, since changes in the cerebral neurotransmitter level could occur in confined brain regions, which potentially could allow for functional imaging of serotonin release. Further, it has been demonstrated in microdialysis studies in rats^{11,12} that the serotonergic response is attenuated by autoregulation from negative feedback of the somatodendritic 5-HT_{1A} autoreceptors. Blocking the autoregulation by pindolol, a 5-HT_{1A} autoreceptor antagonist, thus potentiates the serotonergic response to SSRIs.¹³ In pigs, we have also previously observed a higher serotonergic response to escitalopram in combination with pindolol than in mono-intervention with escitalopram.¹⁵

Although in a different species, our data do not lend support for an acute 5-HT_{1B} autoreceptor-induced decrease in cerebral interstitial serotonin levels upon a single and clinically relevant dose of escitalopram or citalopram as speculated in two human PET studies with [^{11}C]AZ1041936⁷ and [^{11}C]CUMI-101.⁹ The apparently conflicting outcome in the human studies with the non-human primate studies^{6,7,32} and to some extent our pig study may be explained by species differences or differences in study design. Also, it cannot be excluded that the much higher doses of escitalopram used in the non-human primates could induce a larger serotonin release that was more easily detected by PET.

In order to assess the stability of our design, we also investigated the test-retest variability of BP_{ND} in the pig. We demonstrated an excellent reproducibility of [^{11}C]AZ1041936 binding to cortical 5-HT_{1B} receptors with a test-retest variability and ICC comparable to the outcome reported in human studies of [^{11}C]Cimbi-36 binding to 5-HT_{2A} receptors⁴¹ and a little better than that of [^{11}C]SB207145 binding to 5-HT₄ receptors⁴² and [^{11}C]P943 to 5-HT_{1B} receptors.⁴³

A limitation of our study is that microdialysis allows only for measurements in a few selected brain regions, while PET, on the other hand, can generate information about changes in the serotonin level of the entire brain. The low number of pigs in the citalopram and fenfluramine groups also limits the conclusions that can be made for smaller brain regions.

In conclusion, we here show that the [^{11}C]AZ1041936 PET signal correlates to the pharmacologically induced changes in interstitial serotonin brain level compatible with the occupancy model. The observed correlation indicates that [^{11}C]AZ1041936 is sensitive to changes in endogenous serotonin levels, but that is only detectable with PET when the serotonin

release is sufficiently high. The reproducibility of [^{11}C]AZ1041936 is excellent. The sensitivity of [^{11}C]AZ1041936 to detect changes in extracellular serotonin level is comparable to that of [^{11}C]raclopride to detect changes in dopamine level.

Differences in earlier studies may thus be ascribed to the efficacy of the pharmacological interventions to change interstitial brain serotonin levels. Verifying the direct correlation between pharmacologically induced changes in serotonin and [^{11}C]AZ1041936 PET occupancy is an important step prior to conduction of clinical trials and the calibration allows for estimating the regional relative change in interstitial serotonin in patients in future studies.

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Authors' contributions

All authors revised and gave final approval of the manuscript. Following authors contributed to the conception (LMJ and GMK), drafting of the article (LMJ, PW, CS and GMK), design (LMJ, PW, SK, LF and GMK), acquisition of data (LMJ, SK, PW) and analysis and interpretation of data (LMJ, PW, GMK).

Supplementary material

Supplementary material for this paper can be found at the journal website: <http://journals.sagepub.com/home/jcb>

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